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Role of Translocation as a Mechanism of Resistance to Glyphosate

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The continuous use of glyphosate has resulted in the selection of glyphosate-resistant (GR) biotypes in 13 weed species. Decreased translocation of glyphosate to the meristematic tissue is the primary mechanism of resistance in horseweed, hairy fleabane, rigid ryegrass, and Italian ryegrass, and the resistance is inherited as a single, semidominant nuclear trait. The question is: What role does decreased translocation play in glyphosate resistance, and what is the actual mechanism(s)? The enzyme 5-enol-pyruvylshikimate-3-phosphate synthase (EPSPS), the target site of glyphosate, preferentially accumulates in the active meristems of plants. Inhibition of EPSPS results in the accumulation of shikimate. Leaf disc assays across a number of species show that the maximum accumulation of shikimate occurs in young, rapidly expanding tissue. Gene expression studies have also shown that the gene encoding EPSPS is maximally expressed in meristems. Thus, glyphosate needs to translocate to the growing points of plants to be effective. In some GR weed biotypes, glyphosate moves in the treated leaf via the transpiration stream; but instead of being loaded into the phloem, it is trapped in the distal portion of the leaf. These results suggest that there is some type of inhibition of glyphosate-loading into the phloem in GR plants. However, this mechanism may involve uptake of glyphosate at the cellular level. Shikimate accumulation in isolated leaf discs occurs at high glyphosate concentrations in both susceptible and GR biotypes of horseweed and Italian ryegrass; but at low concentrations, shikimate accumulation occurs only in susceptible biotypes. Decreased cellular uptake of glyphosate might occur by one of four mechanisms: (1) the active uptake system no longer recognizes glyphosate, (2) an active efflux system pumps glyphosate out of the cell into the apoplast, (3) an active efflux system pumps glyphosate out of the chloroplast into the cytoplasm, or (4) glyphosate is pumped into the vacuole and sequestered in the cell.
Nomenclature: Glyphosate; hairy fleabane, *Conyza bonariensis* L.; horseweed, *Conyza canadensis* L.; Italian ryegrass, *Lolium multiflorum* Lam.; rigid ryegrass, *Lolium rigidum* Gaud.
Key words: Glyphosate resistance, herbicide resistance, weed resistance, absorption.

Glyphosate is the most widely used herbicide in the world because of the adoption of glyphosate-resistant (GR) crops (Gianessi 2004). Before GR crops were commercialized, there were discussions on the probability of selecting for GR weeds (Bradshaw et al. 1997; Jasieniuk 1995). The argument was that it was unlikely that resistance would be selected by an alteration of the target site, 5-enol-pyruvylshikimate-3-phosphate synthase (EPSPS), because of the high fitness penalty associated with an altered enzyme (Bradshaw et al. 1997). However, in 1997, the first GR weed biotype was reported in populations of rigid ryegrass in Australia. Subsequently, GR weed biotypes and populations have been discovered in 13 species in several countries throughout the world (Heap 2008).

Research on the mechanisms of glyphosate resistance in weeds has revealed that, contrary to prediction, some of these populations are GR because of an alteration of EPSPS. In these biotypes, the Pro at site 106 has been substituted by a Ser, Ala, or Thr, which decreases the ability of glyphosate to bind to the enzyme (Powles and Preston 2006). Although this mutation gives weaker resistance compared with the altered EPSPS in GR crops (Padgette et al. 1996), plants with this mutation can survive when treated at the field use rate.

There is another mechanism of glyphosate resistance that was not anticipated during speculation on whether or not GR weeds would be selected by the continuous use of the herbicide. That mechanism is reduced translocation of glyphosate to the roots and growing points of the treated plant. Reduced translocation is not a common mechanism for herbicide resistance. Only paraquat resistance has been associated with reduced translocation (Fuerst and Vaughn 1990). A biotype of rigid ryegrass that is resistant to glyphosate, paraquat, and acetyl-CoA carboxylase (ACCase) inhibitors has multiple mechanisms of resistance (Yu et al. 2007). Paraquat resistance is due to reduced translocation, whereas glyphosate resistance is due to both reduced translocation and a change of Pro to Ala at site 106 in EPSPS. The ACCase-inhibitor resistance was due to an altered target site. No one has shown differences in the rate or pathway of metabolism of glyphosate between GR and glyphosate-sensitive (GS) biotypes (Powles and Preston 2006; Simarmata and Penner 2008).

The objective of this review is to examine the role of translocation as a mechanism of glyphosate resistance, to speculate on where this mechanism functions in the plant, and to describe the potential mechanism(s). There has been a recent review on the biochemical and genetic basis of glyphosate resistance (Powles and Preston 2006), hence those aspects will not be considered here.

Role of Translocation in Glyphosate Mode of Action

Glyphosate is a potent herbicide because of its ability to translocate in the plant to the apical meristems, root meristems, and underground reproductive organs of perennial plants. Glyphosate kills plants through the inhibition of EPSPS, which is an essential step in the biosynthesis of the aromatic amino acids as well as many secondary plant products (Amrhein et al. 1980).

The genes for EPSPS are most highly expressed in the meristems and flowers of plants, followed by the stem, and

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then by the mature leaves and cotyledons (Weaver and Hermann 1997). Elegant work by Feng et al. (2003) showed that the same tissues in which the genes for EPSPS are highly expressed are also the most sensitive to the herbicide. In velvetleaf (*Abutilon theophrasti* L.), the meristematic zones of the shoots and roots are killed at a tissue concentration of approximately 0.2 mg kg^{-1} of glyphosate, whereas the more-tolerant lower stem tissue required 8.4 mg kg^{-1}.

Inhibition of EPSPS by glyphosate results in the accumulation of shikimate-3-phosphate and shikimate in plant tissue (Amrhein et al. 1980; Schulz et al. 1990). Shaner et al. (2005) compared the rate of accumulation of shikimate in isolated leaf discs across a number of plant species and found that the greatest rate of accumulation occurred in discs taken from the youngest tissue. Mollenhauer et al. (1987) and Schulz et al. (1990) also showed that the highest shikimate accumulation in tomato (*Solanum lycopersicum* L.) leaves occurred in the youngest tissues.

EPSPS, although it is encoded in the nucleus, translocates into the chloroplast, where the aromatic amino acids are synthesized (Schulz et al. 1990). In tomato, glyphosate treatment resulted in the swelling and bursting of chloroplasts, presumably because of shikimate accumulation (Mollenhauer et al. 1987; Vaughn and Duke 1986). Thus, glyphosate needs to enter the cell and then translocate to the active meristems to reach the target site.

Mechanism of Glyphosate Uptake by Plant Cells

The mechanism of glyphosate uptake into plant cells is not well understood. There appears to be at least two mechanisms of uptake, an active system that operates at low concentrations of the herbicide, which may involve a phosphate transporter, and a passive mass flow system (Figure 1). In Madagascar, periwinkle [*Catharanthus roseus* (L.) G. Don], corn (*Zea mays* L.), and soybean [*Glycine max* (L.) Merr.] cell suspensions and in horsebean (*Vicia faba* L.) protoplasts, glyphosate uptake at low concentrations occurred against a concentration gradient and exhibited a saturation phase up to 3 to 50 μM (Denis and Delrot 1993; Hetherington et al. 1998; Morin et al. 1997). The estimated Michaelis constant (substrate concentration at one-half the velocity [K_m]) for the active uptake system was 25 to 31 μM, with a maximum rate of metabolism (maximum velocity [V_{max}]) of 11 to 12.5 nmol g^{-1} fresh wt h^{-1}. Active glyphosate uptake was inhibited by sodium phosphate and phosphonoformic acid, a competitive inhibitor of phosphate uptake in plant cells (Denis and Delrot 1993; Hetherington et al. 1998; Morin et al. 1997). Above 50 μM, glyphosate uptake was linear with external concentrations, and uptake was insensitive to phosphate and to metabolic inhibitors. These data support the existence of active and passive systems of glyphosate uptake.

Efflux experiments in corn and soybean cell suspensions and in horsebean and sugar beet (*Beta vulgaris* L.) leaf discs suggest that glyphosate is located in the cell wall and the cytoplasm, with very little accumulating in the vacuole (Gougler and Geiger 1981; Hetherington et al. 1998; Ibaoui et al. 1986). Gout et al. (1992) also showed that glyphosate was located only in the cytoplasmic compartment of sycamore maple (*Acer pseudoplatanus* L.) cells using phosphorus nuclear magnetic resonance (^{31}P-NMR).

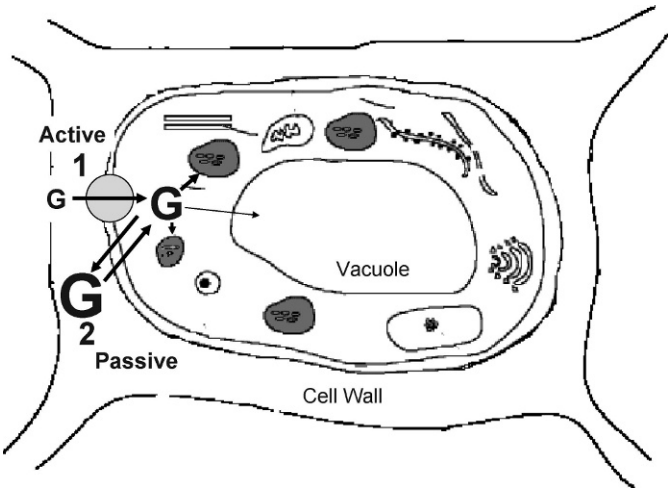

Figure 1. Proposed mechanisms of glyphosate uptake into plant cells. G, glyphosate (the size of the letter indicates relative size of glyphosate pool). (1) Active uptake of glyphosate into cell. (2) Passive diffusion of glyphosate into the cell. Arrows indicate direction of movement of glyphosate pools into and out of the cell, chloroplast and vacuole.

Mechanism of Glyphosate and Photosynthate Translocation in the Plant

The movement of water and sugars through the phloem is due to the active loading of sugars into the sieve elements from the companion cell. The high concentration of sugars in the phloem causes water to enter from the xylem and creates a pressure head, which pushes the water from the source leaves to the sinks (Figure 2) (Christy and Ferrier 1973; Henton et al. 2002).

There are two paths by which sugars enter the sieve elements: the symplasmic and the apoplasmic systems. In plants that load sucrose through the symplasmic system, there are many channels (the plasmodesmata) between the mesophyll cells and the companion cells through which sucrose diffuses. Once the sucrose reaches the companion cells, sucrose is converted to an oligosaccharide (e.g., raffinose). Oligosaccharides cannot diffuse back to the mesophyll cells through the plasmodesmata because of their large size, but the oligosaccharides can be loaded into the sieve element (Lalonde et al. 2003). Plants and genera that have been shown to use the symplasmic system for sucrose loading include, but are not limited to, *Centaurea*, *Impatiens*, *Ligularia*, *Palargonium*, *Pisum*, and *Symphytum* (van Bel et al. 1992).

The apoplasmic system involves a number of sucrose efflux and influx transporters (Figure 2). In plants with this system, sucrose is actively pumped out of the source cells into the cell wall (apoplasmic space), and subsequently, actively pumped into the companion cells. These transporters are driven by the proton motive force generated by an hydrogen ion–pumping adenosine triphosphatase (H+-ATPase) located in the membrane (Lalonde et al. 2003). Genera that have been shown to have the apoplasmic system for sucrose transport include, but not limited to, *Epilobium*, *Fuchsia*, *Hydrangea*, *Oenothera*, *Origanum*, and *Stachys* (van Bel et al. 1992).

Glyphosate is translocated in the phloem from the source leaves to sink tissues following sucrose movement (Gougler and Geiger 1984; McAllister and Haderlie 1985). The phloem mobility of glyphosate is due to its unique

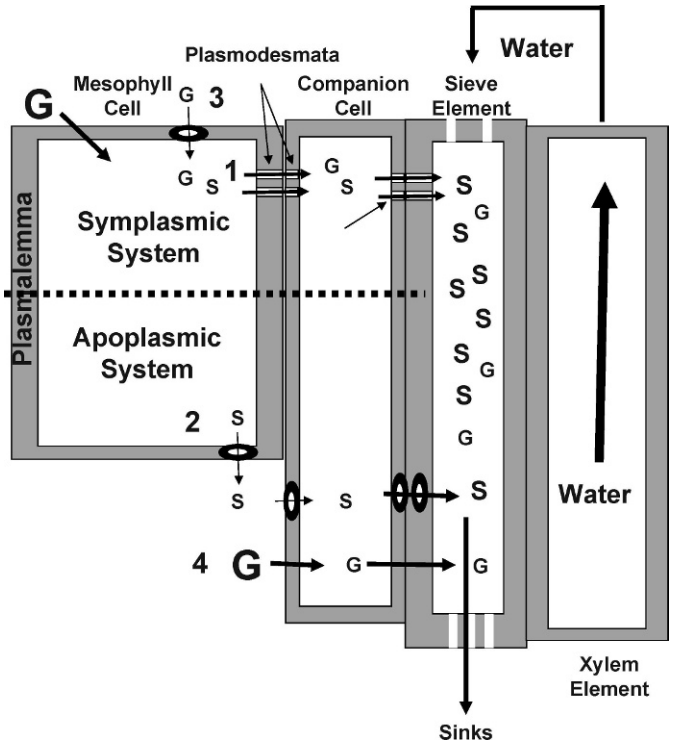

Figure 2. Movement of sucrose, glyphosate, and water into phloem. G, glyphosate; S, sucrose/sugar (size of letter indicates size of pool). Symplasmic system is the cytoplasmic continuous system between cells joined by plasmodesmata. Apoplasmic system is the cell wall and intracellular connections between cells. Grey box is the plasmalemma of the cells. Circles with arrows indicate active transporters, and direction of arrows indicate the direction of the pump (out of cell or into cell across the plasmalemma). Companion cell and sieve element are components of phloem. (1) Symplasmic system: sucrose, produced in mesophyll cell, diffuses to the companion cell via the plasmodesmata. In the companion cell, sucrose is converted to an oligosaccharide that cannot diffuse back through the plasmodesmata but can diffuse into the sieve element. (2) Apoplasmic system: sucrose is actively transported out of the mesophyll cell into the apoplast and is subsequently actively transported into the companion cell via an active influx system. (3) Glyphosate can enter the phloem either via the symplasmic system via an active transporter and then diffuse through the plasmodesmata to the phloem. (4) Glyphosate can enter the companion cell via diffusion.

combination of three acidic and one basic functions (Bromilow and Chamberlain 2000). Any change in the structure of glyphosate that affects its zwitterionic characteristics reduces its ability to move in the plant.

However, it is not known exactly how glyphosate moves into the phloem (Figure 2). The herbicide has to enter the phloem lumen, presumably through the cell symplasm. Glyphosate may do this either by mass diffusion into the mesophyll cells, followed by movement to the phloem through the plasmodesmata, or it may be actively taken into the mesophyll or into companion cells via a phosphate transporter, as described previously. Once glyphosate enters the sieve element, it is trapped because of its hydrophilic properties and is transported to sink tissues.

Low-affinity phosphate transporters, designated as Pht2;1 and Pht1;6, have been identified and are responsible for the movement of phosphate out of source leaves to other parts of the plant (Daram et al. 1999; Rausch and Bucher 2002). Genes encoding these transporters have been isolated from arabidopsis [*Arabidopsis thaliana* (L.) Heynh.], potato (*Solanum tuberosum* L.), and barley (*Hordeum vulgare* L.) (Rae et al. 2003; Rausch et al. 2004). These genes are most highly expressed in the vascular tissue of older, green leaves, and their expression is insensitive to the phosphate levels in the soil, unlike the genes for high-affinity phosphate transporters that are specifically expressed in the roots (Rausch and Bucher 2002). Interestingly, the movement of radiolabeled phosphate from sink leaves to source leaves in potato appears to be similar to the movement of glyphosate in plants (Rausch et al 2004), and this movement is affected by the expression level of the genes for Pht2;1. Could this transporter be involved in the movement of glyphosate?

Glyphosate Self-Limiting Translocation

The amount of glyphosate translocated from source leaves to sink tissues is self-limiting and is due to glyphosate's mechanism of action that inhibits assimilate translocation. In sugar beet, glyphosate inhibition of EPSPS leads to an unregulated flow of carbon into the shikimate pathway and subsequent depletion of carbon cycle metabolites in the dark reaction of photosynthesis (Geiger et al. 1999). This disruption of leaf carbon metabolism inhibits photosynthesis, starch accumulation, and carbon export from the source leaf. The reduction of carbon export from the leaf also reduces glyphosate movement from the source leaves to the meristematic sinks. GR sugar beet cultivars did not show this reduction in either photosynthesis or glyphosate translocation, indicating that the self limiting translocation of glyphosate in GS plants is due to disruption of the shikimate pathway (Geiger et al. 1999).

Glyphosate Resistance due to Limited Translocation

Glyphosate resistance has been selected in multiple weed species, and a major mechanism of resistance appears to be limited glyphosate translocation to the meristematic sinks. This mechanism has been identified in GR biotypes of horseweed (Feng et al. 2004; Koger and Reddy 2005), hairy fleabane (Dinelli et al. 2008), rigid ryegrass (Wakelin et al. 2004), and Italian ryegrass (Perez et al. 2004; Perez-Jones et al. 2007). The pattern of glyphosate movement in these GR biotypes differs from the GS biotypes (Table 1). Less glyphosate translocated out of the treated leaf in the GR biotypes compared with the GS biotypes. These differences resulted in a 3-fold to 10-fold difference in the concentration that inhibits 50% growth (I_{50}) for glyphosate between the GR and GS biotypes.

Mechanisms of Reduced Glyphosate Translocation in GR Biotypes

The mechanisms of resistance attributable to reduced translocation are not known. However, the pattern of glyphosate movement, as well as the accumulation of shikimate, may elucidate potential mechanisms. Glyphosate translocation out of the treated leaf requires that the herbicide enters, and is retained in, the phloem. The different pattern of movement of glyphosate in GR vs. GS biotypes of horseweed, rigid ryegrass, and Italian ryegrass is quite distinct. In GS biotypes, absorbed glyphosate first moves upward in the transpiration stream and is subsequently loaded into the minor veins and moves out of the treated leaf to the rest of the plant. In GR biotypes, glyphosate also moves upward in the transpiration stream, but it does not readily load into the

Table 1. Comparison of glyphosate translocation in glyphosate-susceptible vs. glyphosate-resistant weed biotypes.

Weed	Glyphosate phenotype	Translocation	Glyphosate distribution			Reference
			Treated leaf	Above treated leaf	Root	
		%				
Hairy fleabane	Susceptible	35	63	20	17	Dinelli et al. 2008
	Resistant	16	84	11	7	
Horseweed	Susceptible	30	70	2	20	Koger and Reddy 2005
	Resistant	20	80	1	13	
Italian ryegrass	Susceptible	49	52	37	12	Perez-Jones et al. 2007
	Resistant	26	74	17	9	
Rigid ryegrass	Susceptible	64	37	15	49	Wakelin et al. 2004
	Resistant	53	48	13	40	

minor veins. Hence, less herbicide is translocated out of the treated leaf. In both rigid ryegrass and Italian ryegrass, most of the glyphosate applied to GR biotypes appears to be trapped in the apical portion of the leaf (Perez-Jones et al. 2007; Wakelin et al. 2004). A similar pattern was observed in hairy fleabane and horseweed (Dinelli et al. 2008; Feng et al. 2004). However, with time, the glyphosate in the GR biotypes does enter the phloem and is translocated, although at about half of the amount as in a GS biotype.

These results indicate that there is an unidentified barrier preventing glyphosate loading into the phloem. This barrier could exist in the phloem system or in the mesophyll cells, which glyphosate must enter to be herbicidal as well as to be translocated. Research with isolated leaf tissues suggests that the unidentified barrier actually occurs at the cellular level. In a comparison of GR to GS isolated leaf segments of horseweed and Italian ryegrass, differences were observed in shikimate accumulation at low glyphosate concentrations, but there were no differences in the accumulation of shikimate at high glyphosate concentrations (Koger et al. 2005; Nandula et al. 2008, Perez-Jones et al. 2005) (Table 2). In contrast, there was no accumulation of shikimate at either the low or high glyphosate concentrations in GR soybean.

These results indicate that, at low external concentrations ($\leq$ 10 μM), the amount of glyphosate absorbed by GR leaf tissue is too low to inhibit EPSPS, but at higher concentrations ($\geq$ 250 μM), glyphosate absorbed by the cells is enough to inhibit EPSPS, resulting in shikimate accumulation. One interpretation of these results is that there is an impairment of glyphosate absorption at the leaf cell level that prevents accumulation of glyphosate if the external concentration is low. However, this impairment does not affect the passive, mass-diffusion pathway. Thus, if the external glyphosate concentration is high enough, the herbicide can still enter the cells passively and inhibit EPSPS. Presumably, glyphosate could also diffuse to the sieve element and be translocated to sink tissues, but only at high concentrations.

There are several potential mechanisms by which the cellular absorption and subsequent translocation of glyphosate could be reduced (Figure 3): (1) alteration in a putative phosphate transporter responsible for the active cellular uptake of glyphosate, and if that transporter is no longer present or no longer recognizes glyphosate, uptake and translocation would be reduced at low glyphosate concentrations; (2) evolution of a new transporter that pumps glyphosate into the vacuole, thus sequestering the herbicide and preventing it from reaching either the chloroplast or the phloem; (3) evolution of a new transporter that actively pumps glyphosate out of the cell into the apoplast; or (4) evolution of a transporter at the chloroplast envelope that pumps glyphosate out of the chloroplast, preventing the herbicide from reaching its target site.

There should be methods to determine whether any of these mechanisms exist. If there has been a modification of a phosphate transporter, then cellular absorption of glyphosate should be reduced and measurable either in a disc assay or at the cellular level. There might also be a change in the phosphate levels in GR vs. GS leaves or in the phloem. If glyphosate is being sequestered in the vacuole, then the rate of efflux of glyphosate from the cells in GR plants should be reduced compared with GS plants. On the other hand, if there is a mechanism that actively pumps glyphosate into the apoplast, then there should be an increased glyphosate efflux from GR cells. If the ''pump'' is located at the chloroplast membrane, then measurement of net glyphosate uptake in GR vs. GS chloroplasts would require the isolation of intact chloroplasts. If the mechanism is at the chloroplast level, then there should be no effect on translocation, which appears to be the case in some populations of GR Palmer amaranth (*Amaranthus palmeri* L.) (Culpepper et al. 2006; T. Gaines,

Table 2. Comparison of shikimate accumulation in glyphosate-susceptible vs. glyphosate-resistant weed plants.

Weed or crop	Glyphosate phenotype	External glyphosate concentration[a]		Reference
		Low	High	
		μg g^{-1}		
Horseweed	Susceptible	1,364	1,496	Koger et al. 2005
	Resistant	184	1,424	
Italian ryegrass in Mississippi	Susceptible	600	1,200	Nandula et al. 2008
	Resistant	0	1,200	
Italian ryegrass in Oregon	Susceptible	1,000	1,500	Perez-Jones et al. 2005
	Resistant	0	1,500	
Soybean	Susceptible	784	1,560	Koger et al. 2005
	Resistant	0	0	

[a] Accumulation in the shikimate: low, 5–10 μM; high 250–1,000 μM.

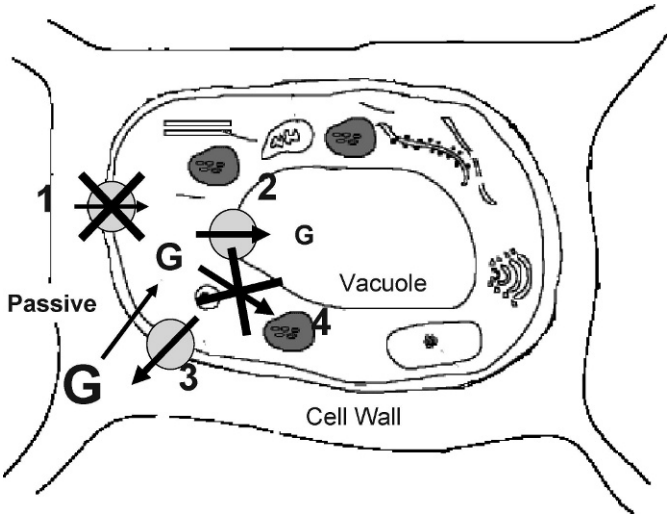

Figure 3. Potential mechanisms for reduced glyphosate cellular uptake in glyphosate-resistant (GR) biotypes. (1) Inhibition of active uptake by a modification of active transporter. (2) An active transporter that pumps glyphosate into the vacuole. (3) An active transporter that pumps glyphosate from the cell into the apoplast. (4) Inhibition of glyphosate uptake into the chloroplast by a transporter that pumps it out of the chloroplast. G, glyphosate.

personal communication). This GR weed biotype shows high tolerance to glyphosate and little accumulation of shikimate. There is no difference in translocation between GR and GS biotypes nor are there any detectable changes in the amino acid sequence of EPSPS that would affect glyphosate binding. However, Simarmata et al. (2003) did not find any differences in glyphosate uptake into chloroplast, isolated from GS and resistant rigid ryegrass biotypes, despite a difference in the amount of herbicide translocated out of the treated leaf between GS and GR biotypes.

There is information that suggests a new transporter may be involved. Adenosine triphosphate (ATP)–binding cassettes (ABC) transporters are membrane-bound proteins that actively transport metabolites into and out of plant cells and vacuoles (Yuan et al. 2007). These transporters are involved in the sequestration of secondary products and herbicide metabolites in the vacuole, the removal of toxic chemicals from plant cells, and the translocation of fatty acids, auxins, and heavy metals. More than 130 genes have been identified that encode for ABC transporters in plants, and it has been reported that there is an ABC transporter gene induced by glyphosate in GR horseweed but not in GS biotypes (Yuan et al. 2007). If GR horseweed has evolved a new, inducible ABC transporter that recognizes glyphosate, then the mechanism of resistance in these biotypes could be due to either sequestration of glyphosate in the vacuole or active pumping of the herbicide out of the cell into the apoplast. This mechanism would result in reduced glyphosate translocation to the meristems.

Glyphosate resistance due to reduced translocation is inherited as a single, semidominant trait in horseweed and rigid ryegrass (Wakelin and Preston 2006; Zelaya et al. 2004). This trait has also been shown to have a fitness penalty. The fitness penalty may be due to a change in the ability of resistant biotypes to absorb or translocate important metabolites or nutrients, such as phosphate. GR rigid ryegrass produced smaller seed compared with a GS biotype, which could be due to less-efficient translocation of photosynthate in the resistant biotype (Pedersen et al. 2007) Importantly, once we understand the mechanisms involved in reduced glyphosate translocation, we may also have a better understanding of the absorption and translocation of nutrients in plants.

Conclusion

The potency of glyphosate is due to excellent translocation to the meristematic tissue of plants, where the shikimate pathway is irreversibly inhibited. Thus, it may not be surprising that reduced translocation is an important mechanism for glyphosate resistance. Although the actual mechanisms that result in reduced translocation or import into target organelles have yet to be elucidated, there are several hypotheses that can be tested. Any, all, or none of the mechanisms proposed in this article may be correct. Elucidating the mechanisms involved in reduced glyphosate translocation will likely lead to a better understanding of metabolite movement in plants.

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